Appendix C. Applicants respectfully request entry of the amendments and remarks made herein into the file history of the present application.

The Rejections Under 35 U.S.C. § 112, Second Paragraph Are Obviated

Claims 22-30 are rejected under 35 U.S.C. § 112, second paragraph as being incomplete for omitting steps relating to dosage and route of administration alleged to be essential. This rejection is obviated by the amended claim which specifies administering an amount of a compound effective to inhibit HBV replication. The amended claim is fully supported by the specification. For example, Sections 5.4.1 and 5.4.2 of the instant specification describes many possible routes of administration and formulations, respectively. Section 5.4.3 of the instant specification describes various compounds and methods that can be used to determine effective dosages to be used in the methods of the invention.

Claim 28 is rejected for recitation of an unclear short abbreviation. In response, Applicants have amended the claim to include the unabbreviated name for BAPTA as suggested by the Examiner.

In view of the foregoing, Applicants request that the Examiner withdraws the rejections under 35 U.S.C. §112, second paragraph.

The Rejections Under 35 U.S.C. § 112, First Paragraph Should Be Withdrawn

Claims 22-30 are rejected under 35 U.S.C. § 112, first paragraph for lack of enablement. The Examiner agrees that the instant specification enables the treatment of a cell line *in vitro* with calcium chelators or calcium modulators to inhibit HBx-mediated activation of Src family tyrosine kinases such as Pyk2. However, the Examiner contends that these data do not enable the use of claimed methods *in vivo*. This rejection is in error and should be withdrawn.

Applicants contend that the Examiner has incorrectly characterized the teachings of the instant specification and has failed to place those teachings in the context of state-of-the-art knowledge. The specification describes methods of treating HBV infection by modulating cytosolic calcium levels. In addition to cell culture data supporting proof of principle, the specification also discloses methods to extrapolate these results to apply therapeutic treatments (see *e.g.*, Section 5.4.3). In particular, the data in the

specification demonstrate that HBx protein is required for HBV replication in cells (see, *e.g.*, Section 7.2 on pages 70-73 of the instant specification), that HBx induces Pyk2 activity (see, *e.g.*, page 79, line 28 to page 80, line 9 of the instant specification), that Pyk2 activity is required for HBx-mediated activation of downstream Src kinase family members (see, *e.g.*, page 80, lines 9-18), and that Src kinase activity is required for HBV replication in cells (see, *e.g.*, page 75, lines 9-33 of the instant specification). The specification further demonstrates that HBx-mediated activation of Pyk2 requires cytosolic calcium (see, *e.g.*, page 80, line 19 to page 81, line 9 and page 82, lines 1-24 of the instant specification). Based on these data, one of skill in the art would recognize that HBV replication could be effectively inhibited by inhibiting HBx action using compounds that modulate cytosolic calcium levels, *i.e.*, that Applicants have successfully demonstrated proof of principle. Apparently, the Examiner agrees.

However, the teachings in the specification go beyond mere proof of principle. Various modalities of treatment using known available compounds are disclosed. One skilled in the art could readily extrapolate dosages and routes of administration using the teachings in the specification (see *e.g.*, Section 5.4.3) to practice the claimed methods of the invention. Applicants point out that the exact formulation, route of administration, and dosage of a calcium modulator according to the present invention will be apparent to the skilled practitioner and should be chosen by the individual physician in view of the patient's condition (*e.g.*, nature and extent of infection, general health, age, etc). Applicants contend that there are a number of calcium channel modulators currently in clinical use for treatment of cardiac disorders (including *e.g.*, those compounds Applicants have disclosed which may be used in accordance with present invention on page 27, lines 8-29). Many of these drugs have been approved for human use prior to the earliest priority date of the instant application (*e.g.*, September 15, 2000) as evidenced by Wood, 1999, *NE J. Med.* 341:1447-57 (attached hereto as Exhibit 1). Thus, one skilled in the relevant art could look to methods used in the determination of administration specifics in this class of drugs to empirically determine compound administration in the methods of the present invention.

Additionally, methods of determining cytosolic calcium levels were also well known in the art prior to the earliest priority date of the instant application. For example, fluorescent and bioluminescent calcium indicator dyes were commercially available from Molecular Probes as of 1999. Chapter 20 of The Handbook of Fluorescent Probes and Research Chemicals published by Molecular Probes (attached hereto as Exhibit 2) details products and methods to determine cytosolic calcium level. Applicants, therefore,

do not need to include a specific dose, route of administration, or method for determining the effect on cytosolic calcium levels of a compound in the claimed methods of the invention as these aspects may be readily extrapolated from the specification.

Contrary to the Examiner's contention (on page 4 of the Office Action mailed September 20, 2002) the use of cyclosporin (for example) to treat HBV infection in accordance with the invention perfectly illustrates why the claims are enabled. Cyclosporin is currently in widespread clinical use and physicians are familiar with dosing regimens used to effectively deal with side effects. The effective doses of cyclosporin taught in the specification are within clinically acceptable and safe ranges. In particular, the specification shows that 1μg/ml of cyclosporin is effective to inhibit HBV replication in cells (see the Example in Section 12 at page 82, lines 1-9 of the instant specification as amended and Figure 10). To achieve therapeutic efficacy, the specification recommends administering cyclosporin to patients in amounts that will achieve such inhibitory plasma levels (see page 46, lines 6-16 of the instant specification). Using ordinary skill, a practicing physician can readily determine the dose required to achieve plasma levels of 1μg/ml. Applicants invite the Examiner's attention to the Physicians' Desk Reference (55th edition 2001, pages 454-461 attached hereto as Exhibit 3) which shows that doses of 7-9 mg/kg/day achieve peak blood concentration (C_{max}) of cyclosporine of 0.66-1.8 μg/ml (see the table at the top of page 455). Thus, one skilled in the art could easily achieve plasma concentrations of cyclosporin taught in the specification for treating HBV (*e.g.*, 1μg/ml) with methods already in use clinically. Because the desired plasma concentrations are within the range of those recited in the Physicians' Desk Reference, one would not expect side effects to be worse than those already deemed acceptable.

A patent applicant's specification which contains a teaching of how to make and use the invention must be taken as enabling unless there is reason to doubt the objective truth of the teachings which must be relied on for enabling support. In re Marzocchi, 439 F.2d 220, 223, 169 USPQ 367, 369 (CCPA 1971); In re Brana, 51 F.3d 1560, 34 USPQ2d 1436 (Fed. Cir. 1995). Here the Examiner can point to no such reason.

Instead, according to the Examiner, although HBx has been studied for a number of years, its true mechanism of action is still unclear. Applicants contend that there is no requirement that the mechanism through which the invention works be known. See Exxon Chemical Patents, Inc. v. Lubrizol Corp. 77 F.3d 450 at 456.

> An inventor need not understand the scientific mechanism in order to place an invention into the patent system. See Newman v. Quigg, 877 F.2d 1575, 1581, 11 USPQ2d 1340, 1345 (Fed.Cir.1989) (observing that "it is not a requirement of patentability that an inventor correctly set forth, or even know, how or why the invention works"); Fromson v. Advance Offset Plate, Inc., 720 F.2d 1565, 1570, 219 USPQ 1137, 1140 (Fed.Cir.1983) ("[I]t is axiomatic that an inventor need not comprehend the scientific principles on which the practical effectiveness of his invention rests.").

It is not important to know the precise mechanism of action that HBx uses to elicit any of its effects during HBV infection - - just that the effects are the consequence of HBx action. As such, one of skill in the art could target the relevant functions of HBx (*e.g.*, activation of Pyk2) to successfully decrease HBV infection or replication without appreciating the full range of HBx activities.

In view of the foregoing, Applicants request that the Examiner withdraws the rejections under 35 U.S.C. §112, first paragraph.

CONCLUSION

Applicants respectfully request that the amendments and remarks made herein be entered and made of record in the file history of the present application. Withdrawal of the Examiner's rejections and a notice of allowance are earnestly requested. If any issues remain in connection herewith, the Examiner is respectfully invited to telephone the undersigned to discuss the same.

Respectfully submitted,

Date: March 20, 2003

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APPENDIX A
MARKED-UP VERSION OF AMENDED PARAGRAPHS
U.S. PATENT APPLICATION SERIAL NO. 09/955,006
ATTORNEY DOCKET NO. 5914-084-999

The Applicants have shown that HBx activation of Src kinases stimulates viral DNA replication, and HBx activates Src kinases by stimulating two related upstream tyrosine kinases known as Pyk2 and p125FAK (FAK). The Applicants have shown that HBx activation of Pyk2, FAK, Src and MAPK signalling, all occur in a calcium-dependent manner in that treatment of cells with calcium chelator (EGTA) or calcium channel poison (bis-(o-aminophenyl)ethyleneglycol-N,N,N',N'-tetraacetic acid or BAPTA-AM) specifically blocks HBx stimulation of Pyk2, which is essential for HBx activity. In addition, treatment of cells with cyclosporin A (CsA), a specific inhibitor of mitochondrial voltage-dependent anion channels, which deregulates calcium channels, also impairs HBx stimulation of HBV genomic DNA replication. Thus, the Applicants have demonstrated that HBx functions through a calcium-dependent pathway to stimulate viral DNA replication in cells and Pyk2 signal transduction, which plays a fundamental role in mammalian hepadnavirus replication.

FIGURE 8. Cells were propagated as described (*Klein, et al.* 1997, EMBOJ 18: 5019-5027), transfected with 5 µg of pΔBS empty plasmid (vector) or pAdCMVX (HBx) expression plasmid (*Klein et al.*, 1997, Mol. Cell. Biol. 17: 6427-6436; *Klein et al.*, 1999, EMBOJ 18: 5019-5027; *Doria et al.*, 1995, EMBOJ 14: 4747-4757), 2 µg of luciferase reporter plasmid controlled by a minimal TATA-box promoter and 4 copies of an AP-1 binding site (*Klein et al.*, 1997, Mol. Cell. Biol. 17: 6427-6436), and 5 µg of PKM plasmid expressing a dominant-interfering form of Pyk2 or pRK5 empty plasmid (*Dikic et al.*, 1996,

Nature 383: 547-550). Cells were allowed to recover for 12 h following transfection, then serum starved for 16 h Chang cells are a human transformed hepatoblastoma line, HepG2 cells are a human differentiated hepatocytic line, and GN4 cells are a rat liver epithelial line. (A) Equal protein amounts were assayed for luciferase activity. For analysis of low level expression of HBx, 0.4 μg of pAdCMVX was transfected. A typical experiment is shown. (B) Cell lysates were prepared in a modified RIPA buffer (*Schlaepfer et al.*, 1998, Mol. Cell. Biol. 18: 2571-2585), gel-electrophoresis and immunoblot analysis was performed with anti-Pyk2 or anti-Y(P)-402 Pyk2 antibodies (Biosource, Int.). Nontransfected cells were treated with 20 ng/ml TPA for 20 mm to activate Pyk2 (TPA samples). (**C**) Equal amounts of protein lysates were immunoprecipitated with antibodies to Pyk2, an in vitro kinase assay was performed using [γ-^{32}P]ATP as described (*Klein, et al.* 1997, EMBOJ 18: 5019-5027), and phosphorylation of associated Src, Fyn, and Pyk2 analyzed by gel-electrophoresis and autoradiography. Identification of Pyk2 and Src-Fyn proteins, which electrophoretically comigrate, was performed by immunoblot with specific antisera (not shown). (**D**) Fyn was immunoprecipitated and autophosphorylation activity (Fyn assay) determined by in vitro kinase assay using [γ-^{32}P]ATP, gel-electrophoresis and autoradiography as described (*Klein et al.*, 1997, Mol. Cell. Biol. 17: 6427-6436; *Klein, et al.* 1997, EMBOJ 18: 5019-5027). Total Fyn protein level was determined by [inimublot] immunoblot of an equal fraction of the immunoprecipitate.

In particular, compounds which may be used in accordance with the present invention to specifically target mitochondrial calcium channels and regulatory components thereof. Compounds which may also be used in accordance with the present invention include those which specifically target

endoplasmic reticulum calcium channels, SERCA Ca^{2+} pumps and regulatory components thereof. Compounds which may be used in accordance with the present invention include: Cyclosporin A, Dihydropyridines: nifedipine (Procardia), nimodipine (Nimotop), amlodipine (Norvasc), felodipine (Plendil and Renedil), isradipine (DynaCirc), nicardipine (Cardene), nisoldipine; Benzothiazepine: [diliazem] diltiazem (Cardizem), Phenylalkylamine , verapamil (Calan and Isoptin), Diarylaminopropylamine ethers, bepridil; Benzimidazole-substituted tetralines , mibefradil Piperazine , flunarizine (Sibelium); (±)-verapamil hydrochloride, omega-Agatoxin TK, omega-Agatoxin Iva, amiloride, Hydrochloride, nimodipine;(±)-Methoxyverapamil, ω-Agatoxin IVA, aminohexahydrofluorene, bepridil, calcicludine, calciseptine, diltiazem, flunarizine, FS2, galanin, HA 1004, HA 1077, nifedipine, nimodipine, nitrendipine, TaiCatoxin, protopine; cyclosporin A; 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetate (BAPTA), BAPTA-AM, MAPTAM, EGTA.

Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the desired modulating effects, or minimal effective concentration (MEC). The MEC will vary for each compound but can be estimated from in vitro data; e.g., the concentration necessary to achieve a 50-90% inhibition of HBV [HCV] infection using the assays described herein. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. However, HPLC assays, bioassays or immunoassays can be used to determine plasma concentrations.

Ras-specific monoclonal antibody Y13-259. (Santa Cruz Biochemicals, Inc.). Rabbit anti-Shc and anti-Sos serum. (Santa Cruz Biochemicals, Inc.). Rabbit anti-Grb2,

anti-ERK2, anti-Csk, anti-JNK and anti-N-Myc were purchased from Santa Cruz. Anti-Src (M327) antibodies were purchased from Oncogene Science. Anti-Fyn antibodies (Santa Cruz) were a gift of D. Littman (NYU). [Anti-phophotyrosine] Anti-phophotyrosine (4G10; UBI) and rabbit anti-p34cdc2 serum and Rabbit anti-RPTPα serum. (Upstate Biotech Inc.).

Studies determined whether HBx acts on intracellular calcium to activate Pyk2. HBx transfected Chang cells showed 5 fold increased phosphorylation of Pyk2 at Y-402, similar to TPA stimulation (Figure 9A). Treatment with the cell permeable cytosolic calcium chelator [BABTA-AM] BAPTA-AM at 50 μM (2 times IC_{50}) for 2 h prevented Pyk2 phosphorylation without altering Pyk2 levels (Figure 9A). HBx activation of Pyk2 therefore involves cytosolic calcium action. Studies next determined whether HBx acts on calcium channels in the endoplasmic reticulum (ER), mitochondria or plasma membrane (PM) for its activity. A low (0.5 mM) concentration of EGTA was added to the culture medium for 2 h to block entrance of extracellular calcium (*Zwick et al.* 1999, J.B.C. 274: 20989-20996), or cells were treated with BAPTA-AM to block ER and mitochondrial calcium, or cyclosporin A (CsA) to block mitochondrial calcium function. EGTA had no effect whereas BAPTA-AM or [GsA] CsA both prevented HBx activation of Pyk2, indicating that HBx acts on [ERimitochondrial] ER/mitochondrial calcium control. A high concentration of EGTA (3 mM) did not block TPA activation of Pyk2 phosphorylation (*Zwick et al.* 1999, J.B.C. 274: 20989-20996) (Figure 9C), but partially inhibited activation by HBx. Therefore, HBx acts on the control of ER/mitochondrial calcium, with low level entry of extracellular calcium, suggestive of constitutive cytosolic calcium alteration (*Clapham* 1997, Cell 80:259-268).

The requirement for cytosolic calcium and HBx activation of Pyk2 in HBV replication was examined. HepG2 cells were transfected with a 130% head-to-tail DNA copy of the HBV genome which replicates authentically in the livers of transgenic mice (*Guidotti et al.* 1995, J. Virol. 69: 6158-6169), in *Tupaja* hepatocytes in culture [**(*Melegari et al.*, 1998, J. Virol. 72: 1737-1743)**] (*Melegari et al.*, 1998, J. Virol. 72: 1737-1743), and in an HBx-dependent manner in HepG2 cells (*Melegari et al.*, 1998, J. Virol. 72: 1737-1743). Expression of HBx was abolished by a targeted frameshift mutation (*Melegari et al.*, 1998, J. Virol. 72: 1737-1743). HepG2 cells were transfected with vector alone, wild type HBV genomic DNA, or HBx(-) genomic DNA, cytoplasmic viral core particles, the structures in which viral DNA replication takes place, were isolated and the level of viral DNA replication was examined (Figure 9D). HBV DNA replication was reduced 20 fold in the absence of HBx expression, but recovered by cotransfection of HBx. Northern [niRNA] mRNA analysis demonstrated no reduction in HBV pregenomic (pg)RNA and HBsAg mRNAs in the absence of HBx (Figure 9D). Cotransfection of wild type HBV genomic DNA with PKM reduced viral DNA replication by 15 fold, similar to HBx(-) HBV samples, without altering viral [niRNA] mRNA levels (Figure 9E). These results demonstrate that HBx specifically promotes HBV DNA replication in a Pyk2-dependent manner.

The requirement for cytosolic calcium in HBx-dependent viral replication was investigated. Cells transfected with wild type or HBx(-) HBV genomic DNA were treated for 4 d with 1 or 3 μg[p.g]/ml of CsA to block mitochondrial calcium channels. There was no evidence for CsA toxicity during treatment. CsA reduced HBV DNA replication in cytoplasmic core particles by 15 fold compared to untreated controls

(Figure 10A), similar in magnitude to inhibition of Pyk2 or the absence of HBx expression. Northern mRNA analysis demonstrated a 2 fold reduction in pgRLNA and HBsAg mRNAs (Figure 10A). To determine whether inhibiting cytosolic calcium and Pyk2 activity inhibits HBV DNA replication, HepG2 cells were transfected with HBV genomic DNA and treated with CsA, or cotransfected with PKM. Cytosolic core particles were purified and incubated with [α-^{32}P]-dNTPs to examine endogenous HBV polymerase activity (Figure 10B). In untreated controls, predominantly full-length double-strand DNA products were produced, indicative of pgRNA reverse transcription and DNA-dependent DNA synthesis. PKM inhibition of Pyk2 or treatment of cells with CsA prevented DNA replication by 7 and 12 fold respectively. Treatment of HBV genome transfected cells with low levels of BAPTA-AM for 4 d impaired viral DNA replication by 10 fold without strongly reducing HBV mRNA levels (Figure 10C). Collectively, these data show that HBx activation of HBV reverse transcription and DNA replication involves alteration of cytosolic calcium and coupled activation of Pyk2. The requirement for cytosolic calcium in HBx transcriptional stimulation was investigated in HepG2 cells transfected with luciferase reporters controlled by transcription factor AP- 1 or CREB, with or without treatment of cells by CsA (Figure 10D). HBx activation of AP- 1 dependent transcription was impaired 2.5 fold by treatment of cells with 10 μg/ml CsA. HBx stimulation of CREB-dependent transcription was resistant to high dose CsA treatment, consistent with HBx activation of CREB by direct interaction (*Andrisani et al.*, 1999, J. Oncol. 15: 1-7). These data indicate that HBx transcriptional activation of AP- 1 but not CREB requires alteration of cytosolic calcium.

APPENDIX B
MARKED-UP VERSION OF AMENDED CLAIMS
U.S. PATENT APPLICATION SERIAL NO. 09/955,006
ATTORNEY DOCKET NO. 5914-084-999

22. (amended) A method for treating Hepatitis B virus (HBV) infection or inhibiting HBV virus replication comprising administering [a compound] to an HBV-infected patient a compound that modulates the level of cytosolic calcium, in an amount effective to inhibit HBV replication.

26. (amended) The method of claim 24 wherein the compound inhibits or interferes with the activity of an [endosplasmic] endoplasmic reticulum calcium channel.

28. (amended) The method of claim 24 wherein the compound is 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetate (BAPTA) or bis-(o-aminophenyl)ethyleneglycol-N,N,N',N'-tetraacetic acid (BAPTA-AM).

APPENDIX C
PENDING CLAIMS AS OF MARCH 20, 2003
U.S. PATENT APPLICATION SERIAL NO. 09/955,006
ATTORNEY DOCKET NO. 5914-084-999

22. (amended) A method for treating Hepatitis B virus (HBV) infection or inhibiting HBV virus replication comprising administering to an HBV-infected patient a compound that modulates the level of cytosolic calcium, in an amount effective to inhibit HBV replication.

23. A method for inhibiting Hepatitis B virus (HBV) replication in a cell wherein the level of cytosolic calcium is altered comprising administering a compound that reduces the altered level of cytosolic calcium to levels comparable to those observed in the absence of HBV.

24. The method of claim 22 or 23 wherein the compound decreases or interferes with an HBx-mediated change in cytosolic calcium.

25. The method of claim 24 wherein the compound decreases or interferes with the activity of a mitochondrial calcium channel.

26. (amended) The method of claim 24 wherein the compound inhibits or interferes with the activity of an endoplasmic reticulum calcium channel.

27. The method of claim 24 wherein the compound is Cyclosporin A.

28. (amended) The method of claim 24 wherein the compound is 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetate (BAPTA) or bis-(o-aminophenyl)ethyleneglycol-N,N,N',N'-tetraacetic acid (BAPTA-AM).

29. The method of claim 22 wherein the compound that modulates the level of cytosolic calcium is determined by an *in vitro* assay comprising:

a) contacting a cell expressing HBx with the compound;

b) determining whether the level of cytosolic calcium is modulated in those cells contacted with the compound as compared to the level of cytosolic calcium in cells expressing HBx in the absence of the compound.

30. The method of claim 23 wherein the compound that alters the level of cytosolic calcium is determined by an *in vitro* assay comprising:

a) contacting a cell expressing HBx with the compound;

b) determining whether the level of cytosolic calcium is altered in those cells contacted with the compound as compared to the level of cytosolic calcium in cells expressing HBx in the absence of the compound.